Hepatic apolipoprotein A-I gene expression in patients with cholesterol gallstones treated with ursodeoxycholic acid
Abstract

Objective: It has been suggested that apo A-I can inhibit cholesterol crystal nucleation in vitro, and ursodeoxycholic acid (UDCA) is a safe and effective treatment for selected patients with cholesterol gallstones the aim of this study was to investigate the effect of UDCA on the steady-state levels (SSL) of apo A-I mRNA in the liver, as well as serum apo A-I, in patients with cholesterol gallstones.

Design: Twenty Mexican patients with symptomatic radiolucent gallstones were randomized and assigned in a double-blind fashion to groups that were administered either UDCA (4 mg/kg per day) or placebo for 10 to 15 days before cholecystectomy.

Methods: Apo A-I mRNA levels in liver and gallbladder tissues were determined by northern blot and serum levels of apo A-I by turbidimetric method.

Results: Apo A-I mRNA levels were higher in nine of the 10 patients who received UDCA and in comparison to those to the placebo group. In the gallbladder apo A-I mRNA levels were undetected. Serum levels (mg/dL) of apo A-I were similar in both UDCA and placebo groups after treatment (111.7 ± 29.8 vs 115.6 ± 25.4).

Conclusions: The results of this study shown that apo A-I mRNA gene express at the mRNA level in the liver but not in the gallbladder of patients with cholesterol gallstones treated with UDCA.

Key words: Apolipoprotein A-I, ursodeoxycholic acid, gallstones, cholesterol, bile acids.

Introduction

It has been suggested that changes to plasma HDL levels commonly reflect altered metabolism of the major HDL apolipoproteins, apo A-I and apo A-II, but the regulation of apolipoprotein metabolism is poorly understood. Although a number of pharmacological and dietary factors may be mediated by these and other transcription factors to affect apo A-I levels, apo A-I gene expression has proven relatively insensitive to physiological change. Some of the states characterized by altered apo A-I gene expression in rodents include experimental hyperthyroidism (1.7–2.5-fold increased apo A-I mRNA), and nephrotic syndrome (2-fold increased apo A-I mRNA).

High fat diets are also associated with a 40% increase in apo A-I synthesis, reflecting increased translational efficiency of apo A-I mRNA. Interestingly, some research has reported that apolipoproteins A-I, A-II, and C-3 can inhibit cholesterol crystal formation in model biles. Furthermore, it has been observed that ursodeoxycholic acid ([UDCA) 3α, 7β, dihydroxy-5β-cholanoic acid] induces an increase in serum apo A-I. UDCA also improves serum liver tests and histological features in a number of chronic cholestatic disorders, including primary biliary cirrhosis and primary sclerosing cholangitis.

In addition, UDCA has been shown to prolong transplant–free survival in patients with primary biliary cirrhosis. The proposed mechanisms of action of UDCA in cholestasis involve the stimulation of impaired biliary secretion, immunomodulating effects, protection against injury of the bile ducts, and antiapoptotic effects. UDCA is also a safe and effective treatment for selected patients with radiolucent cholesterol gallstones. Jüngst et al. have shown that in patients with cholesterol gallstones a low dose of UDCA (250 mg/d) at bedtime for 6–10 days prior to cholecystectomy increases the time to cholesterol nucleation in gallbladder bile. In a similar group of patients, Tazuma et al. reported that the administration of UDCA (300-600 mg/d) induced a significantly longer median nucleation time (16 days) compared with that in patients with cholesterol gallstones who received no preoperative treatment (4 days) (< 0.01). Furthermore, three months of treatment with UDCA significantly elevated the serum concentrations of the antinu-
Patients and methods

Twenty consecutive patients who underwent elective cholecystectomy for symptomatic gallstone disease at the General Hospital of Mexico City were seen in a one-year period. Patients were selected according to the following criteria: functioning gallbladder and radiolucent gallstones, as documented by an oral cholecystogram four weeks before surgery, and a cholesterol content of > 60% in the removed stones. All patients gave their signed consent to participation in the study. Patients with clinical or laboratory evidence of diabetes mellitus, ethanol abuse, hyperlipidaemia, or liver, kidney, or thyroid function abnormalities, were excluded. No patient received oral contraceptive steroids or hormonal therapy. The experimental protocol was approved by the Ethics Committee of the General Hospital of Mexico and The Medica Sur Clinic & Foundation as conforming to the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from all participants before entry. Eligible patients were randomized and assigned in a double blind fashion to groups that were administered either UDCA (4 mg/kg per day) or placebo capsules identical in appearance to those of UDCA (Ursofalk, Laboratorios Farmasa, S.A. de C.V. Mexico City) 10-15 days before cholecystectomy. Medication was well tolerated, and there were no side effects. Liver function tests and body weights remained unchanged during the whole treatment period. Patients were hospitalized in the Gastroenterology Surgical Unit, two to three days before surgery, and were fed, preoperatively, the standard hospital diet (carbohydrates 60%, fats 20%, proteins 20%).

All surgical procedures (open cholecystectomies) were performed between 08: 00 and 10: 00 am, after a 14-h fasting period. A wedge liver biopsy (100-400 mg) was routinely taken from the right lobe of the liver, frozen immediately on dry ice and acetone, transported to the laboratory, and stored at -70°C until the extraction of total RNA. Stones were removed from the gallbladder, washed with a solution of 0.9% NaCl, and the cholesterol content analysed by X-ray diffraction.12 Immediately after removal of the gallbladder, samples of gallbladder tissue (15 x 15 mm) were taken from the neck, body, and fundus, and were frozen and stored as indicated previously.

Isolation of total cellular RNA

Total cellular RNA was isolated according to the method of Chomcsynski and Sacchi, by acid guanidium thiocyanate-phenol-chloroform extraction.13 Approximately 100 mg tissue was minced and homogenized with 2 mL denaturing solution (solution D) (4 M guanidinium thiocyanate, 25 mM sodium citrate, [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). The homogenate was then transferred to a 15 mL Corex tube, and 0.2 mL 2 M sodium acetate (pH 4.0), 2 mL phenol at 65°C, and 0.4 mL chloroform were added. The final suspension was shaken, and cooled on ice for 15 min. Samples were centrifuged at 10,000 g for 20 min at 4°C, and the RNA in the aqueous phase was precipitated with 0.25 M sodium chloride and 2.5 volumes of ethanol. The pellets were resuspended in 0.4 mL solution D, and the phenol-chloroform extraction was repeated. The RNA was then dissolved in 200 µL TE buffer (10 mM Tris-HCl [pH 7.0], 1 mM EDTA).

Northern Hybridization Analysis of Total RNA

Total RNA (10 µg) was denatured for 15 min at 60°C in buffer containing 50% formamide, 6% formaldehyde, and MOPS buffer (20 mM 3-(N-morpholino) propanesulfonic acid, 5 mM sodium acetate, 1 mM Na2-EDTA), and samples were loaded into separate lanes of a 1% agarose gel prepared in MOPS buffer with 6% formaldehyde. Samples were separated electrophoretically for 3-4 h at 60 mA, essentially as described previously.14 After electrophoresis, RNA was transferred to Gene Screen membrane (New England Nuclear Corp., Boston, MA, USA), according to the manufacturer’s instructions, and hybridized with a radiolabelled probe. An apo A-I clone15 was radioactively labelled by primer extension, as described by Summers,16 using 32P-dCTP (3000 Ci/mmol) to a specific activity of 2-6 x 10^6 cpm/µg DNA. After hybridization, filters were washed and exposed to autoradiography at –86°C on Kodak XAR-5 film, using Dupont Lightening Plus intensifying screens. Finally, specific mRNA bands were analysed densitometrically, as described previously.17

Serum lipids and apolipoprotein A-I measurement

Serum cholesterol and triglyceride levels were determined in both groups, and total serum cholesterol concentration was measured before and after treatment, using the monotest cholesterol kit provided by Laboratories Lakeside (Mexico).18 Serum triacylglycerol concentrations were determined using an enzymatic method.19 Triacylglycerols were completely hydrolysed, and the liberated glycerol measured by colorimetry. HDL were separated from VLDL and LDL by precipitation with phosphpontusrc acid.20 LDL cholesterol was precipitated by adding polyvinyl sulphate to the sample, and the concentration was calculated from the difference between total cholesterol and the cholesterol in the supernatant after centrifugation. HDL and LDL cholesterol were determined as described previously.18 VLDL cholesterol was calculated as the difference between total plasma and (LDL + HDL) cholesterol.21 The accuracy of HDL and LDL measurements...
were verified using control sera, as indicated by the manufacturer (Laboratories Lakeside). Serum apo A-I levels were determined in both groups, before and after treatment, by a turbidimetric method.22

**Statistical Analysis**

All results are expressed as the mean ± SD or median and range. Mann-Whitney U test and Student’s unpaired test were used for group comparisons, with the level of statistical significance set at \( p < 0.05 \).23

**Results**

The clinical characteristics of patients were similar in both the UDCA and placebo groups: mean age (years) 43.6 ± 14.4 vs 38.9 ± 10.5, respectively, and body weight (kg) 62.2 ± 6.1 vs 63.3 ± 9.4, respectively.

**Steady-state levels of apo A-I mRNAs**

The steady-state levels of hepatic apo A-I mRNA increased in nine of 10 patients after UDCA therapy (Figure 1), but in none of the placebo group. Studies performed on gallbladder tissue (neck, body, and fundus) in both groups of patients failed to reveal apo A-I mRNA (Figure 2).

**Serum Apolipoprotein A-I**

Basal serum levels (mg/dL) of apo A-I were similar in both UDCA and placebo groups (110.7 ± 13.5 vs 126.9 ± 29.1), and were not significantly different after treatment (111.7 ± 29.8 vs 115.6 ± 25.4). However, we found an increase in serum levels of apo A-I in three of nine patients (in another patient, the liver sample was insufficient to measure apo A-I) who presented with an increase in SSL of apo A-I mRNA (Table I).

**Serum Lipids and Lipoproteins**

*Table II* summarizes the serum levels of lipids (cholesterol and triglycerides) and lipoproteins (HDL, LDL, and VLDL). No significant differences were observed between UDCA and placebo groups, before or after treatment, except in serum levels of triglycerides in the placebo group, which were lower in patients after treatment (140.10 ± 47.81 vs 107.20 ± 53.48), therefore becoming significantly different from the UDCA values.

**Discussion**

In this study, we have observed that patients with cholesterol gallstones treated with low doses of UDCA (4 mg/kg per day) for a short period (10 to 15 days) showed higher hepatic transcription of the apo A-I gene. However, apo A-I protein was not increased in the serum of all patients with high levels of hepatic apo A-I mRNA. Similar results have been observed under different pathophysiological conditions in experimental animal models of liver disease,24 suggesting postranscriptional regulation of apo A-I expression. However, the question arises whether an increase in the expression of the apo A-I gene at the mRNA level has a beneficial effect on liver function, as well as on the biliary tract. As mentioned above, apo A-I appears to play a role as an antinucleating factor in model bile. This effect is probably important in preventing cholesterol gallstones. Swell et al.25,26 have suggested that the apo A-I present in human gallbladder bile is derived mainly from the liver. Interestingly, Poynard et al.27,28 have reported that, among drinkers, apo A-I and apo A-II serum concentrations are closely related to the degree of liver injury, reaching a maximum in patients with steatosis, decreasing in patients with fibrosis, and at a minimum in patients with severe cirrhosis. Furthermore, the prevalence of cholesterol gallstone disease in patients without fibrosis (normal liver or steatosis) is low, at 5% and 6%, respectively. In contrast, in patients with fibrosis or severe liver disease, gallstone occurrence increases to 13% and 25%, respectively.27 In another prospective study on 67 male patients with cirrhosis, gallstone disease was found in 37% of the patients. The occu-
rence of gallstone disease was not related to age, weight, or the severity of liver disease. Total serum bilirubin was higher and apo A-I levels were lower in those patients with gallstones. Apo A-I was the only factor associated independently with the occurrence of gallstones. It is important to mention that patients with chronic hepatic diseases usually develop pigmented gallstones, and the pathogenesis of these types of stones is different from that of cholesterol gallstones. However, in both types of stones the bile was saturated with cholesterol or bilirubin, and the role of antinucleating factors such as apo A-I may be very important. In contrast, Otha et al. detected a decrease in the distribution of apo A-I in the liver and biliary tree in patients with intrahepatic cholesterol and brown pigmented stones. Interestingly, Pattnion et al. have identified apo A-I, apo A-II, and apo-B in normal and pathologically affected human bile ducts and in the gallbladder epithelium, using an avidin–biotin immunoperoxidase technique. They suggest a role for these apolipoproteins in the gallbladder epithelium. However, in those two publications, the researchers identified apo A-I by using an immunohistochemical technique that is less specific than the northern blot used in this study. The immunohistochemical technique is also known to produce cross-reactions.

### Table I. Effects of UDCA treatment on apo A-I gene expression at the mRNA and serum levels.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Hepatic mRNA levelsa (Liver tissue)</th>
<th>Serum protein levels (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>1</td>
<td>4.5</td>
<td>132</td>
</tr>
<tr>
<td>2</td>
<td>3.8</td>
<td>118</td>
</tr>
<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>1.8</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>92</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>7</td>
<td>2.3</td>
<td>118</td>
</tr>
<tr>
<td>8</td>
<td>6.0</td>
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</tr>
<tr>
<td>9</td>
<td>5.8</td>
<td>104</td>
</tr>
<tr>
<td>10</td>
<td>N.D.</td>
<td>97</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>110.7 ± 13.5</td>
<td>111.7 ± 29.9</td>
</tr>
</tbody>
</table>

a Arbitrary units; N.D., not determined; b not statistically significant.

### Table II. Serum lipids and lipoproteins in patients treated with a low dose of UDCA.

<table>
<thead>
<tr>
<th></th>
<th>UDCA</th>
<th>Placebo</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>171.2±53.7</td>
<td>146.4±37.2</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>51.9±47.5</td>
<td>107.2±53.5</td>
<td>0.04</td>
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<tr>
<td>LDL</td>
<td>35.3±7.9</td>
<td>33.4±5.5</td>
<td>NS</td>
</tr>
<tr>
<td>HDL</td>
<td>108.0±48.5</td>
<td>92.0±28.5</td>
<td>NS</td>
</tr>
<tr>
<td>VLDL</td>
<td>25.8±8.6</td>
<td>21.0±10.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoproteins; NS, not significant.

Secknus et al. have proposed a purified 15 kDa protein, derived from the whole apo A-I-bound fraction, as a novel potent biliary crystallization inhibitor protein. The same group of investigators reported that apo A-I in bile has a direct effect on cholesterol crystal formation, and enhances lipid removal from gallbladder bile by gallbladder epithelial cells. These observations support two separate roles for human biliary apo A-I, and suggest that this protein may be important in preventing the formation of cholesterol crystals (the initial step in gallstone formation) in supersaturated bile. Recently, Ginanni Corradini et al. proposed that one of the main defects in cholesterol gallstone patients occurs at the gallbladder mucosa. They found, using an in vitro-isolated intra-arterially perfused gallbladder model, that human gallbladder epithelium loses its capacity to selectively and efficiently absorb cholesterol and phospholipids from bile. These observations suggest that this defect is not only relevant at the level of lipid absorption, but may also involve other aspects of epithelial function, such as an increased secretion of mucin or other proteins that promote cholesterol crystallization and decreased secretion of anticrystallization proteins such as apo A-I.

On the other hand, experiments have suggested that UDCA increases bile flow in the rat, and that apo A-I is transported to the bile at the canicular level. Furthermore, Van Erpecum et al. studied the gallbladder bile of 13 patients with cholesterol gallstones who were treated with UDCA (10 mg/kg per day), and compared them with 13 untreated patients. They found higher concentrations of total protein in the gallbladder bile of UDCA-treated patients than in untreated patients (6.7 ± 1.3 vs 2.8 ± 0.6 mg/mL, respectively; p = 0.008) and similarly higher concentrations in the concanavilin A-binding fraction (0.42 ± 0.07 vs 0.16 ± 0.03 mg/mL, respectively; p = 0.003). They concluded that UDCA greatly reduces the levels of various proteins and has nucleation-promoting activity in bile.

Finally, in humans, low HDL cholesterol levels may be associated with defects in the synthesis or catabolism of the major HDL apolipoprotein, apoA-I, with catabolic defects being more common. Low HDL levels are often accompanied by hypertriglyceridaemia, obesity, and insulin resistance, and these alterations are often seen in patients with cholesterol gallstones. These data suggest that apo A-I plays an important role in the pathogenesis of cholesterol gallstones.

In conclusion, the results of this study shown that apo A-I mRNA gene express at the mRNA level in the liver but not in the gallbladder of patients with cholesterol gallstones treated with UDCA.

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